Somatic Mutations in the Peutz-Jegners (*LKB1/STKII*) Gene in Sporadic Malignant Melanomas

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Germline mutations in the *LKB1/STK11* gene cause characteristic hamartomas and freckling to develop in patients with Peutz–Jeghers syndrome (PJS). The hamartomas arise as a result of somatic "second hits" at *LKB1/STK11* and therefore contain a neoplastic element. The origin of the pigmented lesions in PJS is unknown and difficult to test, as these are hardly ever biopsied. PJS patients are at increased risk of benign and malignant tumors, particularly of the colon, breast, pancreas, testis, and ovary, although the increased risk for any one of these sites may be quite modest. Somatic *LKB1/STK11* mutations have been found, albeit at a low frequency, in sporadic tumors of the colon, stomach, ovary, and testis. Although PJS patients are not known to have an excess of skin tumors, if the freckles of PJS patients are actually small, benign tumors, *LKB1/STK11* mutations must provide these lesions with a selective advantage, and similar mutations might also give a selective advantage to related malignant tumors, such as melanomas. We have therefore screened 16 melanoma cell lines, 15 primary melanomas, and 19 metastases for *LKB1/STK11* mutations. Two *LKB1/STK11* mutations were found: a missense change (Y49D) accompanied by allele loss in a cell line; and a missense change (G135R), without a detected mutation in the other allele, in a primary tumor. Both these mutations are highly likely to be pathogenic. Novel polymorphisms, including an unusual heptanucleotide repeat, were also found in introns 2 and 3. *LKB1/STK11* mutations occur in a significant minority of tumors of several sites, including malignant melanomas. *Key words: LKB1/STK11/melanoma/Peutz-Jeghers. J Invest Dermatol 112:509–511, 1999*

Germline mutations in the *LKB1/STK11* gene (chromosome 19p13.3) cause Peutz–Jeghers syndrome (PJS, MIM175200) (Hemminki *et al*, 1998; Jenne *et al*, 1998). PJS has pathognemonic features of multiple gastrointestinal hamartomas, which have an arborising structure and smooth muscle core, and melanin freckling, which is usually present on the lips and inside the mouth, and may also occur on the digits, palms, soles, and axillae (reviewed in Tomlinson and Houlston, 1997). *LKB1/STK11* acts as a tumor suppressor gene in the hamartomas of PJS patients (Hemminki *et al*, 1997).

PJS patients are at about 20-fold increased risk of benign and malignant tumors, particularly of the colon, breast, pancreas, testis, and ovary, although the increased risk for any one of these sites may be quite modest (Tomlinson and Houlston, 1997). By analogy with hamartoma predisposition genes such as *PTEN* and *DPC4*, *LKB1/STK11* is a good candidate for involvement in the pathogenesis of a spectrum of sporadic cancers. Mutations of *LKB1/STK11* have been found in a significant minority of sporadic tumors of the colon (Dong *et al*, 1998; Wang *et al*, 1998; Avizienyte *et al*, 1998), ovary (Wang *et al*, 1999), stomach (Park *et al*, 1998), and testis (Avizienyte *et al*, 1998).

The origin of the pigmented lesions in PJS is essentially unknown. It is generally supposed that they are simple lentigines, but this theory does not explain why PJS freckles are relatively highly pigmented or why they occur in areas that usually show no freckling at all and/or have little light exposure. An alternative theory is that the PJS freckles are actually benign neoplasms of melanocytes (or some precursor), which have a very limited growth potential and do not seem to increase in size throughout life (Tomlinson and Bodmer, 1996); indeed, PJS pigmentation often fades in middle age. This theory is extremely difficult to test, because PJS freckles are hardly ever biopsied, but is consistent with data showing that PJS pigmentation can occur in proliferative lesions such as psoriatic plaques (Banse-Kupin *et al*, 1986).

There are no reports of any PJS freckle becoming malignant, but if the freckles of PJS patients are actually small, benign tumors, *LKB1/STK11* mutations must provide these lesions with a selective advantage. Although PJS patients are not known to be at increased risk of skin tumors (Boardman *et al*, 1998), it is theoretically possible that *LKB1/STK11* mutations provide a selective advantage to malignant lesions that are related to PJS freckles, e.g., malignant melanoma. There are few, if any, reports of allele loss (LOH) close to the *LKB1/STK11* locus on 19p13.3 in melanoma, but this may

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Abbreviation: PJS, Peutz–Jeghers syndrome.

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reflect an absence of studies in this region and translocation breakpoints have been observed in melanomas in this region (Parmiter *et al*, 1986). Below, we report the results of screening 16 melanoma cell lines, 15 primary melanomas, and 19 metastases for somatic *LKB1/STK11* mutations.

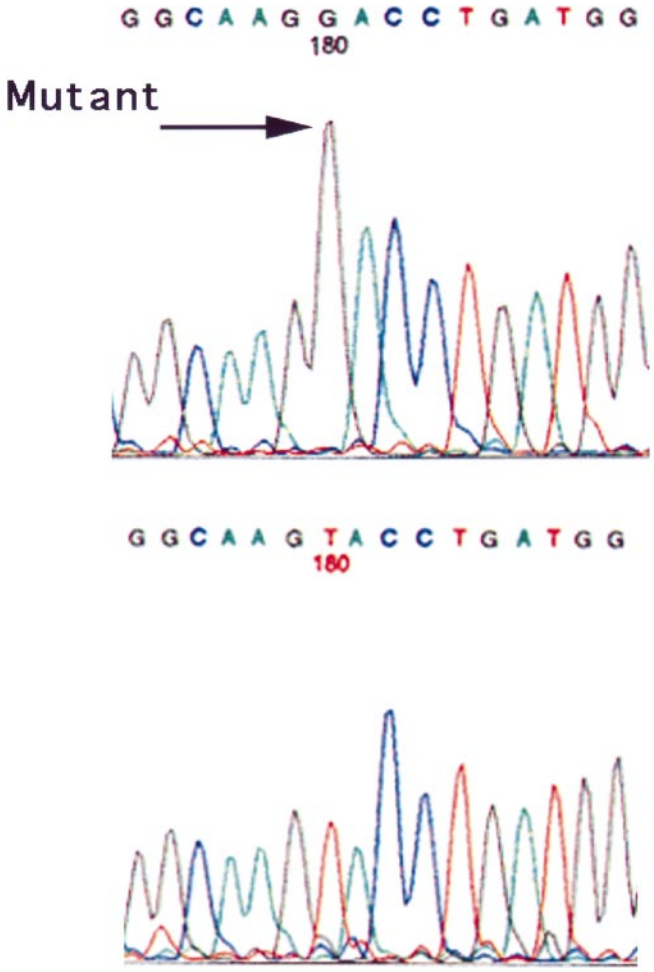

Figure 1. Y49D LK*B1*/STK11 mutation in melanoma cell line WDS3. The mutation is arrowed in the tumor sample (above). Note the absence of the underlying wild-type (T) allele in the tumor, compared with the normal sequence (below).

MATERIALS AND METHODS

Melanoma tissue was derived from either cells (16 cell lines), fresh-frozen tumors (19 metastases), or microdissected, paraffin-embedded, archival material (14 primary tumors and one metastasis). None of these cases had known clinical or familial features suggestive of PJS, consistent with the fact that melanoma is not a tumor associated with PJS. DNA was extracted from each of the tumors to be studied using the Qiagen Tissue Extraction Kit. Standard clinicopathologic data (patient age, tumor grade, and stage) were available from hospital records.

Single strand conformational polymorphism analysis was performed on the tumor samples in order to screen for mutations in the coding regions and flanking intronic sequences of *LKB1/STK11*. Published oligonucleotides and reaction conditions (Wang *et al*, 1998) were used for exon-by-exon amplification of *LKB1/STK11* in the polymerase chain reaction. Polymerase chain reaction products were heated to 90°C for 5 min and subjected to electrophoresis on a 12.5% precast acrylamide gel under denaturing conditions using the Phast System (Pharmacia, Uppsala, Sweden). DNA was detected by silver-staining of gels using the standard Phast System protocol. For all tumors with possible mutations according to single strand conformational polymorphism analysis, that exon was reamplified from genomic DNA in the polymerase chain reaction and purified polymerase chain reaction products were sequenced in forward and reverse orientation using the ABI Ready Reaction Dye Terminator Cycle Sequencing kit and the 377 Prism sequencer. All sequencing reactions were performed in duplicate and alongside samples with wild-type genotypes and patient samples with known germline mutations in *LKB1/STK11*.

RESULTS AND DISCUSSION

We found a mutation of *LKB1/STK11* in two of 50 (4%) melanomas studied. One of these tumors was a cell line derived from a primary lesion and the other was from a primary melanoma; in neither case was constitutional DNA available for analysis. Both mutations are novel missense variants (**Figs 1**, **2**). In the cell line, Tyr is substituted for Asp at codon 49. In the primary lesion, Arg is substituted for Gly at codon 135. Both changes are highly likely to have pathogenic effects, because: (i) the amino acid changes are nonconservative, substituting a charged for an uncharged residue; (ii) no polymorphism at either site has been reported previously by any group; (iii) the mutations lie at the start of or within the kinase core of the *LKB1/STK11* protein (Hemminki *et al*, 1998); (iv) both the amino acids concerned are conserved between human, mouse, and *Xenopus* and lie within conserved motifs (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=3024672&form=6&db=p&Dopt=f; http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=3024670&form=6&db=p&Dopt=f; Churchman *et al* unpublished data); (v) we have found no germline variant at either site in over 150 patients with tumors and over

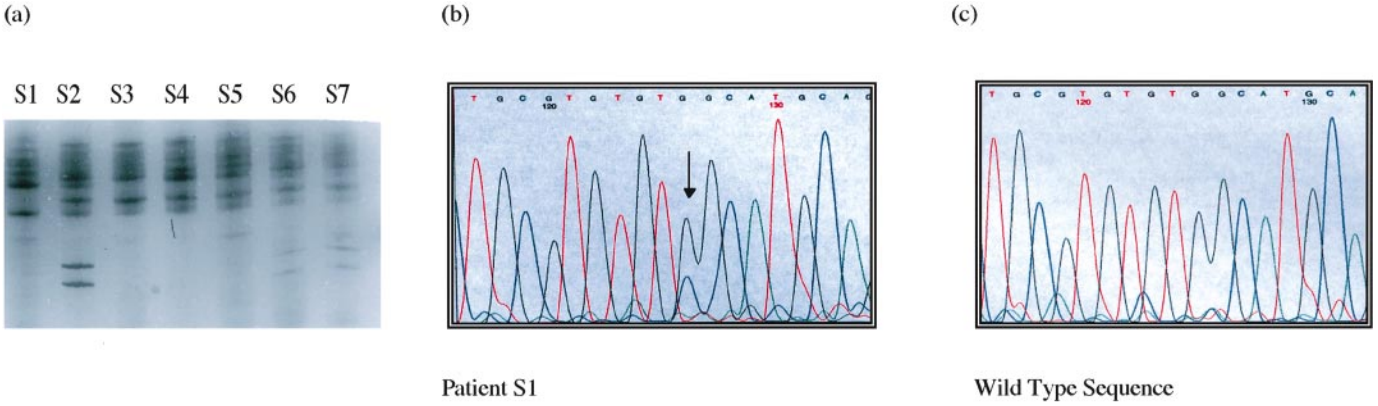

Figure 2. G135R LKB1/STK11 mutation in primary tumor S1. (*a*) Single strand conformational polymorphism analysis of tumor (S1) for exon 3 (*lane S1*); tumors without exon 3 mutations are shown in the other lanes of the gel. Note the additional bands in *lanes S2*, *S6*, and *S7*, which result from the C/G SNP polymorphism at positions +49 and +50 in intron 3. (*b*) Sequence of the mutation (*arrowed*). Note that the mutant allele (C) in tumor A is weaker than the wild type (G). This probably results from the presence of a small amount of contaminating normal tissue in the tumor specimen. This mutation is repeatedly detectable and inspection of the surrounding sequence shows no spurious peaks masquerading as missense changes. The change in tumor A does not affect a restriction site and the mutatution cannot, therefore, be confirmed using this method. The wild-type sequence is shown in (*c*).

50 normal individuals screened previously; and (vi) in the case of the cell line, no wild-type *LKB1/STK11* allele was detected on sequencing (**Fig 1**), strongly suggesting mutation of the other allele by loss of heterozygosity. No "second hit" was found in the melanoma with the G135R mutation, but the action of *LKB1/STK11* as a tumor suppressor (Hemminki *et al*, 1997; Park *et al*, 1998) strongly suggests that a second mutation (or an alternative such as promoter methylation) had occurred in this tumor.

Three novel polymorphisms were detected in introns 2 and 3 of *LKB1/STK11.* These comprise an A/G single nucleotide polymorphism (SNP) at the −49 position in intron 2; G/C SNP at both +49 and +50 of intron 3; and an unusual heptanucleotide repeat (with alleles of two and three repeat units) at +30 of intron 3. In addition, a previously described common missense polymorphism was detected in intron 7 (Wang *et al*, 1998). Formal assessment of allele frequencies at each site was not undertaken. Combined use of these polymorphic sites may be useful for further studies of *LKB1/STK11.*

Although our method is sensitive enough to detect common missense polymorphisms (see above), it is unlikely that single strand conformational polymorphism has 100% sensitivity for point mutations. Moreover, we have not searched for large-scale deletions of *LKB1/STK11* or for promoter methylation. The involvement of *LKB1/STK11* in melanoma pathogenesis may therefore exceed 4% of tumors.

The accumulated evidence suggests that *LKB1/STK11* mutations are involved in the pathogenesis of a small but potentially significant minority of sporadic malignant melanomas, in addition to cancers of the stomach, ovary, and testis. Neither of the tumors with *LKB1/STK11* mutations in our study had exceptional clinical features. The frequency of *LKB1/STK11* mutations in colon cancer is probably similarly low (Wang *et al*, 1998; Avizienyte *et al* 1998), although one report suggests that mutations occur in as many as 54% of left-sided colon cancers (Dong *et al*, 1998). The mutations in most of the tumor types are too rare for any associations between the molecular and clinicopathologic data to be detected. *LKB1/STK11* appears to act as a tumor suppressor in sporadic tumors, as it does in the hamartomas in PJS (Hemminki *et al*, 1997). It is noteworthy that the majority of somatic *LKB1/STK11* mutations in sporadic tumors are missense changes (Dong *et al*, 1998; Avizienyte *et al*, 1998; Park *et al*, 1998), suggesting that abolition of kinase function is not required to provide tumors with a selective advantage. By contrast, most, although not all, germline *LKB1/STK11* mutations lead to a truncated protein (Hemminki *et al*, 1998; Jenne *et al*, 1998; Nakagawa *et al*, 1998).

The question remains of whether or not the characteristic freckles of PJS patients are (clonal) neoplasms. This hypothesis can be proved by finding allele loss or other somatic *LKB1/STK11* mutations in the freckles themselves, but none of over 100 PJS patients that we have studied has ever had a freckle biopsied. We have analyzed a biopsied oral freckle from a patient with typical PJS freckling, but no known gastrointestinal hamartomas, and have found no evidence for *LKB1/STK11* mutations.

One of the unexplained phenomena in Mendelian cancer syndromes is the site specificity of cancers caused by mutations in genes with apparent "housekeeping" roles. One particular aspect of this problem is that germline mutations in cancer-predisposing genes often produce a very different tumor spectrum from the spectrum of tumors that carry somatic mutations in the same genes. Unusually, the spectrum of cancers in the inherited syndrome of PJS seems to be as broad as the spectrum of sporadic cancers that have somatic mutations in *LKB1/STK11.* The combined data on *LKB1/STK11* mutations in sporadic tumors accords with the relatively low excess site-specific risk of cancer that is found in PJS patients. The relatively infrequent occurrence of *LKB1/STK11* mutations in sporadic tumors also suggests some genetic redundancy in the pathways of tumorigenesis, but the large variety of inherited and sporadic tumors with *LKB1/STK11* mutations suggests a widespread role for normal *LKB1/STK11* in areas such as preventing tumor growth.

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REFERENCES

Avizienyte E, Roth S, Loukola A, *et al*: Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. *Cancer Res* 58:2087–2090, 1998

Banse-Kupin LA, Douglass MC: Localization of Peutz-Jeghers macules to psoriatic plaques. *Arch Dermatol* 122:679–683, 1986

Boardman LA, Thibodeau SN, Schaid DJ, *et al*: Increased risk for cancer in patients with the Peutz–Jeghers syndrome. *Ann Intern Med* 128:896–899, 1998

Dong SM, Kim KM, Kim SY, *et al*: Frequent somatic mutations in serine/threonine kinase 1/Peutz–Jeghers syndrome gene in left-sided colon cancer. *Cancer Res* 58:3787–3790, 1998

Hemminki A, Tomlinson I, Markie D, *et al*: Localization of a susceptibility locus for Peutz–Jeghers, syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. *Nat Genet* 15:87–90, 1997

Hemminki A, Markie D, Tomlinson I, *et al*: A serine/threonine kinase gene defective in Peutz–Jeghers syndrome. *Nature* 391:184–187, 1998

Jenne DE, Reimann H, Nezu J, *et al*: Peutz–Jeghers syndrome is caused by mutations in a novel serine threonine kinase. *Nat Genet* 18:38–43, 1998

Nakagawa H, Koyama K, Miyoshi Y, *et al*: Nine novel gennline mutations of STK11 in ten families with Peutz–Jeghers syndrome. *Hum Genet* 103:168–172, 1998

Park WS, Moon YW, Yang YM, *et al*: Mutations of the STK11 gene in sporadic gastric carcinoma. *Int J Oncol* 13:601–604, 1998

Parnliter AH, Balaban G, Herlyn M, Clark WH Jr, Nowell PC: A t(1;19) chromosome translocation in three cases of human malignant melanoma. *Cancer Res* 46:1526–1529, 1986

Tomlinson IPM, Bodmer WF: Failure of programmed cell death and differentiation as causes of turnors: some simple mathematical models. *Proc Natl Acad Sci USA* 92:11130–11134, 1996

Tomlinson I, Houlston R: Peutz–Jeghers syndrome. *J Med Genet* 34:1007–1011, 1997

Wang Z-J, Taylor F, Churchman M, Norbury CG, Tomlinson IPM: Genetic pathways of colorectal carcinogenesis rarely involve the PTEN and LKB1 genes outside the inherited hamartoma syndromes. *Am J Pathol* 153:363–366, 1998

Wang Z-J, Churchman M, Campbell IG, *et al*: Allele loss and mutation screen at the Peutz-Jeghers (*LKB1*) locus (19p13.3) in sporadic ovarian tumours. *Br J Cancer* in press, 1999